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The Patent Office

Cardiff Road
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1. Your reference GBP86709

2. Patent application number
(The Patent Office will fill in this part) 0225390.4

31 OCT 2002

3 Full name, address and postcode of the or of each applicant (underline all surnames)

Horticulture Research International,
Wellesbourne
Warwick
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United Kingdom

Patents ADP number (if you know it) 62605440001

If the applicant is a corporate body, give the country/state of its incorporation United Kingdom

4. Title of the invention SELECTIVE EXPRESSION IN FILAMENTOUS FUNGI

5. Name of your agent (if you have one)
"Address for service" in the United Kingdom to which all correspondence should be sent (including the postcode)

Marks & Clerk
57 - 60 Lincolns Inn fields
London WC2A 3LS

Patents ADP number (if you know it) 18001

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Country	Priority application No (if you know it)	Date of filing (day / month / year)

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Number of earlier application	Date of filing (day / month / year)

8. Is a statement of inventorship and of right to grant of a patent required in support ofthis request? (Answer 'Yes' if:
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SELECTIVE EXPRESSION IN FILAMENTOUS FUNGI

The present invention relates to the transformation of filamentous fungi with heterologous DNA, methods for such transformation and processes for the collection of the resulting expression products.

Filamentous fungi have an unusual life cycle, in that the majority of the cycle is spent as mycelium, which is a largely hidden network of filaments permeating throughout and over a suitable food source. In the case of *Agaricus bisporus*, or button mushroom, which is a horticultural adaptation of a wild mushroom, the food source is decomposed leaf litter, in the wild, or generally composted cereal straw, when cultivated, and little signs of growth can be observed above ground level, other than the characteristic smell, until the fruiting bodies, or sporophores, start developing.

Compared with the culture of other vegetables, the commercial production of button mushrooms is complex. They are best grown indoors in controlled environmental conditions in trays, shelves or bags filled with a speciality compost, specifically made by, and for the, the mushroom industry from ingredients including: plant remains, such as cereal straw, corn husks, hay, rape straw; animal manure, such as from horse, chicken, cow, pig; gypsum, and other additives.

Mushroom compost is made by the mixing of the raw ingredients, for example straw, manure and gypsum base, with water, which leads to microbial degradation and heat production. The composting process has two phases. In Phase 1 composting, the mixture is either stacked in a windrow outdoors or in a barn and turned every 2 to 3 days over a three-week period, or it is placed in a walled bunker and aerated from below over an 8-10 day period. This compost is then subjected to Phase II composting in an aerated bunker where the temperature is raised to 58-60°C for approximately 12 hours and held at 50-54°C for 5 days. The compost is then ready for spawning.

Spawn is, typically, sterilised cereal grain, such as rye or millet, which has been colonised with the mushroom mycelium. Spawn is mixed with the compost and maintained at 24°C. After approximately 17 days at this temperature, the compost is fully colonised by the mycelium.

A casing layer is then applied to the surface of the colonised compost to a depth of approximately 5 cm. Casing normally consists of peat (or peat substitute for example, coir, bark, mineral washing), chalk/lime/sugarbeetlime, water and a small amount of specialised spawn, which may be the mycelium cultured on compost, grain, vermiculite or other substrate. When the surface of the casing layer is colonised with mycelium, the temperature is lowered to 16-18°C, which induces production of mushroom Sporophores, or fruiting bodies. From application of the casing to harvesting the first sporophore takes approximately 17 days.

The production of mushrooms occurs periodically, over cycles known as flushes, at 7-10 day intervals. High productivity farms normally crop mushrooms over 3 flushes. The time from the first harvest of the first flush to the last harvest of the third flush is about 21 days.

The time each growing house is occupied with each crop is from after casing and during crop production, or 17 + 21 = 38 days, or thereabouts. This allows a growing house to be used for 9 crops per year. High productivity farms routinely have yields of 25kg/m^2. Because mushroom production is not light dependent, the crops are grown in layers, either four or six to maximise productivity. Accordingly, the four-layer system is expected to produce 3,200 tonnes per annum in a hectare of growing rooms.

The rapid development of the fruiting body is unique in the vegetable kingdom, and represents a significant target for the expression of suitable genes. If this rapid, volumetric expansion of the fruiting body could be harnessed together with expression of a suitable gene, then this might make possible the production, on an industrial scale, of biological products that currently defy large scale production.

Attempts to express heterologous proteins in filamentous fungi have met with numerous problems, not least being the difficulty associated with transforming the fungi in the first place. For example, WO95/02691 relates to transforming a mushroom mycelium and fruit bodies through methods such as electroporation with suitable vectors. Although this method works on a small scale, it is not especially efficient.

WO98/45455 relates to the possibility of transforming moulds, such as *Agaricus bisporus*, with the *Agrobacterium tumefaciens* bacterium, which causes crown gall tumours at the wound site of infected dicotyledonous plants. This bacterium is well known for its ability to transform plants, but it has only recently been established that it can also transform filamentous fungi.

The problem still remains that the expression of heterologous genes in substantial amounts will generally substantially reduce the growth potential of the mycelium and, therefore, the harvest of the fruiting body, and prohibits the expression of any substance which is, in any way, toxic to the growth of the fungus.

Surprisingly, it has now been found that at least three genes are switched on, or otherwise subjected to elevated levels of expression, at around the veil-break stage of fruiting body development, and that heterologous DNA under the control of the expression mechanisms of these fungal genes can be selectively expressed at this stage of development of the fungus, rather than during growth of the mycelium.

Thus, in a first aspect, the present invention provides a filamentous fungus transformed with a heterologous sequence of DNA, the fungus being capable of expressing the heterologous DNA, characterised in that the heterologous DNA is under the control of a filamentous fungus transcription promoter active substantially only during stage 1, or later, of the development of the fruiting body of the fungus.

It is a particular advantage of the present invention that little or no metabolic energy need be diverted from mycelium growth, thereby maximising fruiting body mass and concomitant tissue capable of expressing the heterologous gene once it is switched on.

It is a further advantage of the present invention that little or none of the heterologous gene product is expressed during vegetative growth of the mycelium, thus enabling the production of substantially any substance capable of expression in the filamentous fungus in question, even if that substance, either alone or in combination, results in the death or stasis of the fungus. The promoters allow the synchronous switching on of the gene at a time of rapid growth and high metabolism so that, by the

time any potentially toxic effects become apparent, harvestable quantities of the substance are available. Where the gene product is a regulator of mushroom growth, for example, then such considerations are not generally necessary.

At present, no less than three genes have been identified that are expressed substantially only during development of the fruiting body, and particularly during stages 4 to 7 (veil-break onwards). Without being constrained by theory, it is likely that these genes are associated with the massive water uptake required for the expansion of the fruiting body and its maturation.

The three genes, so far identified, are *abst1*, *rafe* and *mag2*. The expression product of *abst1* appears to be involved in the transport of sugars, whilst the expression product of *rafe* is a putative riboflavin aldehyde forming enzyme. The expression product of *mag2* is a so far unidentified morphogenesis associated protein.

The sequences of the three genes, and associated promoter and terminator regions, are given in the accompanying Sequence Listing, whilst Figure 1 illustrates the stages of development of the fruiting body of a mushroom fungus.

Figure 2 illustrates the level of expression of *abst1* during the various stages of development of the fruiting body, whilst Figure 2A illustrates the gene expression associated with the various parts of the fruiting body at stage 4.

Figure 3 corresponds to Figure 2, except showing the expression of *rafe*, whilst Figure 4 shows the expression of *mag2*.

It will be appreciated that the Basidiomycetes, including members of the *Agaricales*, of which *A. bisporus* is one, share the exceedingly rapid development of the fruiting body in common. Without being constrained by theory, it is envisaged that this development is as a result of a rapid increase in osmotic pressure in the cells of the immature fruiting body, thereby causing a rapid influx of water into the cells. The resulting sudden expansion of the cells expands the fruiting body up to several hundred times its original size.

One or more sugar transport mechanisms are switched on at the early stages of fruiting body development, and abundant expression product is noted, especially by stage 4. These genes form a preferred subject of the present invention, and especially the control element associated therewith, but it will be appreciated that any gene selectively expressed, or with greatly enhanced expression, during development of the fruiting body is useful in the present invention.

The *abst1* gene is up-regulated, by more than 100-fold, during mushroom development, and is abundantly expressed through stages 4 to 7, and represents about 0.6% of the transcripts detected at stage 4. The transcript is about 1.7kb in length.

The gene product of *rafe* is up-regulated by about 50-fold during mushroom development, and is abundantly expressed from stage 4 onwards, as with *abst1*. The transcript is 0.7kb in length.

The expression product of *mag2* is up-regulated by about 30-fold during mushroom development, and represents about 0.2% of the transcripts at stage 4. Unlike *abst1* and *rafe*, the expression of *mag2* appears to be comparable in both the stipe and cap tissues. The length of the transcript is about 0.7kb.

The control elements, and especially the promoters, of these and other genes expressed during the development of the fruiting body are particularly useful in the present invention. Elements from genes associated with sugar transport are particularly preferred.

It is a particular advantage of the present invention that heterologous genes can be expressed at selected stages of sporophore development, where these genes might otherwise be harmful to the fungus. Expression of the heterologous genes occurs substantially only during growth of the fruiting body so that, unless the gene product is acutely toxic, then large amounts of the gene product can be expressed which would otherwise harm or hinder growth of the fungus.

Owing to the commonality of the fruiting body growth process, the promoters of any one filamentous fungus, switched on during the fruiting body growth cycle, may be

employed in other filamentous fungi in the context of the present invention, in order to express heterologous genes.

In the accompanying Sequence Listing, it will be appreciated that specific promoter sequences have not been identified. However, the full sequences are provided for *abst1* and *rafe*, including a substantial portion of the upstream promoter area and, following the procedures described in the accompanying Example, the upstream promoter area is also obtainable for *mag2*. Although not critical to the present invention, the same also applies to the terminator regions.

The promoter regions may be used in their entirety when preparing heterologous genes for expression in filamentous fungi. Alternatively, it may be preferred to use consensus sequences from these regions. There is no especial advantage to using consensus sequences, except that these may be shorter. Otherwise, it is sufficient to supply the promoter upstream of the desired heterologous gene. Being a promoter, there is also no requirement that it be in the correct reading frame, just within the appropriate promoter distance.

The promoter may be used in association with other suitable control sequences, such as terminators. A suitable terminator may be as shown in the accompanying sequences, or may be the Aspergillus nidulans trpC terminator, for example. Other terminators are well known in the art.

It is generally preferable that the transformed fungus also expresses a linked selectable marker. Any marker known in the art may be used, and may be excised once a faithful strain has been generated. However, it is generally preferable that the transformed fungus maintains a marker to ensure that the desired heterologous product is still produced, and to ensure that there is no reversion to wild type. In this respect, it is preferred that the marker have no significant negative effect on either the fungus or the product. Such markers may normally be selected from resistance markers, in order that the growth medium contain amounts of an antifungal agent ensuring that only transformed fungus can grow successfully.

Suitable markers include the hygromycin resistance cassette and the benomyl resistance tubulin gene.

Suitable methods for transforming filamentous fungi are as described above with respect to WO95/02691 and WO98/45455, which disclosures are incorporated herein by reference.

In general, the desired control sequences are ligated with the appropriate heterologous expression sequences and prepared for insertion into a suitable preparation of the fungus, such as protoplasts, all by methods well known in the art.

The resulting organism can then be grown by standard methods, and prepared as spawn after cultivation of the resulting mycelium. Spawn has the advantage that it can be stored inert for relatively long periods of up to about a year, although it is generally preferred to use it within about 4 months.

Spawn may be produced in any recognised manner, such as by growing the mycelium on sterile agar and introducing the culture to autoclaved grain. The grains may then be stored at elevated temperature to encourage colonisation, and then kept at reduced temperatures until needed.

It will be appreciated that the heterologous gene for incorporation may be in the form of cDNA or genomic DNA. cDNA is preferred, as it is generally shorter and more easy to handle.

It will also be appreciated that the heterologous gene insert should encode the sequence desired, including leader sequences and cleavage sequences, if required.

It will also be appreciated that greater expression may be achieved if fungal codons are used in place of mammalian codons, although expression will still occur, and such substitution is not necessary.

It will also be appreciated that heterologous genes may need to be expressed in the form of a cassette, for example, in order to produce the required product. In general, it is preferred to require as few heterologous gene products as possible, as the greater

the number, the more likely it is that the fungal metabolism will interfere in some way, and it is generally desirable to minimise unpredictability.

Thus, although not limited thereto, it is generally preferred to limit the number of heterologous expression products to one, two or three, preferably one or two, and preferably one, other than any marker. The marker is preferably linked to the heterologous gene, such as downstream of the gene and also under the control of the fungal promoter, so as best to indicate successful and/or continuing stable transformation.

It is particularly preferred that products such as peptides be the target, as these can be harvested relatively simply. Thus, enzymes and antibodies are particularly useful, although conformational proteins, such as vaccine antigens, and active peptides such as interferons are also useful.

Accordingly, heterologous genes suitable for expression in the filamentous fungi include those whose expression results in the production of: antibodies, including other diagnostic material; secondary metabolites, such as lectins, pesticidal compounds such as *Bacillus thuringiensis* toxin (Bt toxin); therapeutic compounds such as vaccines, steroids, heterocyclic organic compounds; biological macromolecules, such as interferon, endostatin and insulin; and medical enzymes, such as thrombolytics and cerebrosidases.

In the context of the present invention, the term "heterologous" includes native DNA not normally associated with heightened expression during sporophore production. In such a respect, the native gene becomes heterologous insofar as its expression pattern is altered. Such expression may generally serve one of two purposes. The first is generally to obtain large/greater amounts of native protein, such as by transforming the filamentous fungi with extra copies or modified copies of a native gene or genes. The second may be used instead to affect/control the characteristics of mushroom crop production, such as by altering the timing of crop, flushing pattern, yield, growth rate and/or final size of the mushroom sporophore. This latter may also suitably be achieved by the introduction of heterologous DNA from other species, if desired.

The crops are preferably allowed to go to full cap development, where possible, in order to maximise expression of the heterologous gene, although the skilled person will appreciate the best stage for harvesting any given product. The resulting caps may then be processed in any suitable manner to extract and/or purify the product, or the caps may otherwise be employed or processed, as desired.

Where the product is potentially dangerous, standard procedures may be employed between crops to entirely sterilise the area, such as steam sterilisation and swabbing of the walls, as described above.

The present invention will now be illustrated further, by reference to the following, non-limiting Example.

EXAMPLE

Mushroom strains and growing conditions

A commercial *A. bisporus* strain U3 (Sylvan, U.K.) and a carboxin resistant *A. bisporus* mutant C54-*carb*.8 were used in this work. Vegetative mycelium was produced on sterile compost at 25°C and fully colonised compost (21 days) was frozen in liquid nitrogen. Mushrooms were grown in trays according to commercial practice at the Horticulture Research International mushroom cropping unit. Sporophores were produced in synchronous weekly flushes and mushroom fruit bodies from second flush were harvested at developmental stages one to seven and flash frozen. For tissue expression analysis, stage 4 mushrooms were dissected into stipe (upper and lower), cap (pileus trama), skin (pilei pellis) and gills (lamellae) and frozen. All frozen samples were stored at –80°C.

Bacterial strains, vectors and phagemid/cosmid DNA extraction

Escherichia coli strains XL-1 Blue and XLOLR (Stratagene) were used for the preparation and propagation of cDNA clones. Phagemid and Cosmid DNA extractions were carried out using the Tip 20 plasmid DNA extraction kit (Qiagen).

Preparation of an ordered library of random cDNA clones

Total RNA extraction and poly $(A)^+$ RNA isolation were carried out as described previously. Using 5 µg poly $(A)^+$ RNA, cDNA libraries (ZAP EXPRESS, Stratagene) were constructed from veil break stage mushrooms, and mushrooms were harvested and stored for two days. Mass excision of the cDNA libraries was performed according to the manufacturer's (Stratagene) instructions. Clear single colonies (cDNA clones in pBK-CMV) were picked and ordered in microtitre plates containing 200 µl media 96 broth with Kanamycin (50 µg ml^{-1}) in each well.

Differential screening

Duplicate nylon membranes containing DNA from the clones were prepared for differential screening. For preparing probes cDNA was generated from 5 µg poly $(A)^+$ RNA using the Ready-to-Go T-primed cDNA synthesis kit (Amersham Pharmacia Biotech). Total cDNA's were labelled with [α-^{32}P] dCTP using the Redi-prime random labelling kit (Amersham Pharmacia Biotech). One set each of the membranes containing 3500 cDNA clones from the veil break stage mushrooms were hybridised with the cDNA probe from the veil break stage mushrooms and the cDNA probe from button stage mushrooms. Putative differentially expressed cDNA clones were re-screened to reduce false positives.

Northern analysis

Total RNA was extracted from compost colonised vegetative mycelium and seven different developmental stages of the mushroom, freshly harvested button stage mushroom and samples stored from 1 – 5 days as well as from the stipe, cap and gill tissue. Equal quantities (15 μg) of total RNA for each sample were electrophoresed and Northern blots prepared as described elsewhere. cDNA randomly labelled with [α-^{32}P] dCTP, as described above, was used as the probe.

Genomic library screening and identification of the gene

For isolating cosmid clones containing the gene, a genomic library constructed from a carboxin resistant mutant of *A. bisporus* C54-*carb*.8 in cosmid vector Lawrist was used. Preliminary screening of DNA pools from 56 microtitre plates (96 clones each, *ca.* 5376) was done by PCR using primers designed from the cDNA sequence. Individual cosmid clones containing the gene were identified by probing colony blots of the 96 clones in each of the positive pools.

Nucleotide sequence determination and analysis

cDNA's in the phagemid vector pBK-CMV were initially sequenced using the vector primers T3 and T7 and the full double strand sequence was obtained using additional primers synthesised from known sequence. Genomic sequence was generated from cosmid clones by primer walking, where the initial sequencing was by done using the cDNA primers and further primers for sequencing were designed from known sequence. Nucleotide sequences were determined using the ABI automated DNA sequencing technology. Sequencing reactions were carried by thermal cycling using the ABI Prism™ BigDye terminator cycle sequencing kit (ABI-Perkin Elmer) as per manufacturers' instructions. Editing and assembling of the sequence data were done

using the programmes within the DNASTAR package (Lasergene software, Dnastar Inc.). Homology searches and nucleotide and amino acid sequence comparisons were made using a suite of software available on the WWW, particularly ExPASy (www.expasy.com) and other linked sites.

SEQUENCE LISTING

Organism:	*Agaricus bisporus*
Gene designations (tentative):	*abst1* (sugar transporter); *rafe* (putative riboflavin aldehyde forming enzyme gene); *mag2* (unidentified morphogenesis associated gene)
Gene identification:	By differential screening of a cDNA library (3500 random clones) from stage 4 mushrooms
Genomic clones:	From a genomic library of *A. bisporus* strain C54-*carb*8 in cosmid vector Lawrist (*ca.* 30 – 40 kb genomic fragments)
abst1:	Up-regulated (more than 100 fold) during mushroom development, abundantly expressed through stages 4 – 7 (later stages of mushroom development), *ca.* 0.6% transcripts at stage 4, represented by 20 clones in the differential library, 1.7 kb transcript
rafe:	Up-regulated (up to 50 fold) during mushroom development, abundantly expressed through stages 4 – 7, 0.7 kb transcript
mag2:	Up-regulated (up to 30 fold) during mushroom development, *ca.* 0.6% transcripts at stage 4, represented by seven clones in the differential library, comparable levels of expression in stipe and cap tissue, ca. 0.7 kb transcript

1. *abst1*:

cDNA sequence:

GGCTGAGCTCTATTCATCATGGCGTCGGAACGACAGATTGAAGAACTTCC
CAGTTATAAGTACGCCTATATTTTGACGGCATCGGCTTGCTTGGGAAGT
GTGTTTCACGGGTGGGATGTAGGCCTTATAGGAGGCATACTCTCGCTA
CGGTCATTTCAAGAATATCTCGGGATCAATACAAAAAATGCCGTCAAGA
AAGCGATTCTAGACGGAAACATCATCTCTGTGCTCCAAGCCGGATGTTT
TTTTGGCGCGCTTGGAACCGGATATCTCTCTAGTCGATTCGGCCGAAGA
CCCTGTCTTATTGCATCTGGTATTGTGTATATAACTGGCGGTTTGCTGC
AATGCACTGTCGGTTTGGGACCCTCGCAAGCTGCTGCTCTACACGTGTT
CTATATTGGCAGGTTCATTTCTGGTATCGGTGTTGGGATGGTGTCCACT
CTCGTGCCTTTGTATATTTCGGAGTGTGTCCCTAGGACTATACGCGGGC
GCTGTACTGGAACACTCCAATTTGCGACTAACAGTGGTCTGATGCTGG
GCTTCTGGGTCAACTACAGCGTGTCGAAAAACGTGCCCTTTGGTGAAAT
GCAATGGCGAATTCCGTTAATTATCCAAATGATTCCGAGCCTCTTGTTC
ATCATAGCCATGTTTTTCCAACCAGAATCGCCGAGATGGCTTGTTGAAC
ACGGGAAACACAAGGAAGCTGCGACGGTACTGGCGCGTACTGGCGGCA
AGGATGTTGATCATCCTAGTGTTGTACAGACACTGGAGGAGATCAAGC
AAGAATTTGTGGCGAGTAAACAACCATCGTTTTTAAAGCAGATTCGCCT
GGTCGGTGAATCGAGGGCTGTTGCCCTGAGGTGCTTTATACCACCGCT
AGTGATGTTCTTCCAGCAGTGGACGGGTACAAATGCCATCAACCTTTAT
AGTCCCGAAGTATTCCGTCATCTTGGAATCCATGGCACCAGCGGGGCT
CTCTTCGCTACTGGTGTTTATGGCGTGGTGAAGGTTGTTTCAGTTGCAC
TTGCCCTCACTTTTGCTGTCGAACGCTTTGGACGCAAGAGAGGGTTGAT
TTTTGGTGGTATCGGCCAAGCACTTATGATGTTTTGGTTGGGAGGTTAT
AGTGCCACCCACCAAGACGGTACTGTCAGTCCTGCGAGTCATGTTTCCA
TTGTTGCACTCTACTTGTATGGTGCATTCTTCTCCATGGGATGGGGCCC
ATTACCATGGGTCGTCGCTGGAGAGGTTGCACCTAACCATGTCCGCTC
CTTCGCCCTCTCCATCGCCGTTGGAACTCATTGGCTCTTCGGGTTTGTG
ATATCAAAAGTGACGCCAATTATGTTGGACCGTATCAAATATGGCACAT
TCCTACTCTTCGGATTCTGTTGCATGATAGTAGCGACATGGGCTTATTT
CTGTCTACCTGAGACAAGTGGGTTCGCTCTGGAGGACATCAAATATCTG
TTCGAGCGAGACGTCATCATTCGTTCATTGCAGGACGCTCCCGGTGGA
AAAATATTCTTGGGGGGGAGGCGTGTGGAATCTGTAGCTTCGTTGAAA
GAGAGGCGCGTTGGAGTCGCTGGTGAGCAGGGTGAGAAGATAACTGGT
CTAAATTCGGAATTGGAAGATGTTTCCTCAAAAAAATCAACATTGAAGG
AAACTTCATCCGTTTGATATATAGTCTCCAAATTCTATTGTAATGCCATTTT
CCCAATTCAAAAAAAAAAAAAAAAAAA

Open reading frame coding for predicted protein highlighted.

Genomic sequence:

GTNCGATGGGTTCNTCTGGGTTAAGTTGCACGACGCTTTCCTTTTTCTTTTAT
GGCCTGTCTGCCCTTTTAACGCTTTATCTTTCGGCAGCCATGGATGTCCTTCG
TCACCGTATTATCACTCTTAATCGTGGTGGTGAGCACATGGAAAGGTTCATT
ATCGTCATCCCATGACGCGGTGCAAAATTCGTCATTCAGAGTGGAACCGAT
ACTAGGAGAGGATTTTGAAAAGGCTATCGTATCTTCCGATCCATTCACTCCA
ACGACATCTATATCGGGTTCTCAAGCTACGATAACCGTTCCTCACGCATCAC
ATTCTTCTCTGATTGTAATGACCGAAGACCGCGGTTCCACCCCTAATATTCG
AAGAGCTTGTTGCCAATGTCAAAAAAGGCTTTTGGGCAAACCGGGATGGCT
TATCGAGCCTCCAGCCCCAGCAGCTAACATCGGGCAGAGAAGGAAAATTCA
TCGGGCGGTTGAATTATCACCGTTTGGTTCCTGAGTCATCTGNRAGATGTAC
GCAGATGGTGATACCGTGTTTGATTGGCGCCGTTGGAGAAGAACTATATTAT
TCGATGGATTTTTTGTTCGAGTTTGACACAGAGACAGAGATGATAGAGGTTT
GCTATTGATGTAGCAAAGGATCATTTGACGATGGCGCATAGGGCGATGGTT
ATCTTTATGTCTGGAATTATAATATGTATTGTTCCCCACTTTTCTTTTATATTT
ATTAATACTAATTGGAAGTTTCAGTTGTTGGATGAGCAAAGTTGGTGCAGAT
AGAAACTAGAATTCGGATTCCCATATCTGAGGTACCTTTTCCTTCCGCTGGC
AATCCTGGCCACTTCGACGTGGTGACGCAGAGGGCGCGTGCTATTGTTAGC
ACATGCCATAWGGATCGACGTTGCCTCTCGTACTTCGCGCCTAGGCTCGCTC
ATGCCTCGATGCATCTTTTCAATTCGGGCGTTGCGTCTCCCAGGTGCCTGTT
AAAAGGGCGAACTTTAGTGTAATTGTACTAACACAGTCCCTCGGGCTGAGC
TCTATTCATCATGGCGTCGGAACGACAGATTGAAGAACTTCCCAGTTATAAG
TACGCCTATATTTTGACGGCATCGGCTTGCTTGGGAAGTGTGTTTCACGGGT
GGGATGTGCAAGTATCCTTTWGACGCTGGCTCATCCCTTGTTTGCTGATGGT
TAATGTGTAGAGGCCTTATAGGAGGCATACTCTCGCTACGGTCATTTCAAGA
ATATCTCGGGATCAATACAAAAAATGCCGTCAAGAAAGCGATTCTAGACGG
AAACATCATCTCTGTGCTCCAAGCCGGATGTTTTGTAAGTCGTAGCACTCGT
TCGACCAGCTTACTTTCTTTTACTAACAACGTGTTGACCTAGTTTTGGCGCGC
TTGGAACCGGATATCTCTCTAGTCGATTCGGCCGAAGACCCTGTCTTATTGC
ATCTGGTATTGTGTATATAACTGGCGGTTTGCTGCAATGCACTGTCGGTTTG
GGACCCTCGCAAGCTGCTGCTCTACACGTGTTCTATATTGGCAGGTTCATTT
CTGGTATCGGTGTTGGGATGGTGTCCACTCTCGTGCCTTTGTATATTTCGGA
GTGTGTCCCTAGGACTATACGCGGGCGCTGTACTGGAACACTCCAATTTGCG
ACTAACAGTGGTCTGATGCTGGGCTGTAAGTGTACCTTTTGTTATGCTCCGG
GGACGATACTAAAAGTAGTCTGTAGTCTGGGTCAACTACAGCGTGTCGAAA
AACGTGCCCTTTGGTGAAATGCAATGGCGAATTCCGTTAATTATCCAGTACG
TTATTGGATGCAAAGTGAAGCATATGCTGAAATTAGACTTGTGTCGTACTTT
CCAGAATGATTCCGAGCCTCTTGTTCATCATAGCCATGTTTTTCCAACCAGA
ATCGCCGAGATGGCTTGTTGAACACGGGAAACACAAGGAAGCTGCGACGGT
ACTGGCGCGTACTGGCGGCAAGGATGTTGATCATCCTAGTGTTGTACAGAC
ACTGGAGGAGATCAAGCAAGAATTTGTGGCGAGTAAACAACCATCGTTTTT
AAAGCAGATTCGCCTGGTCGGTGAATCGAGGGCTGTTGCCYTGAGGWGCTT
TATACCACCGCTAGTGATGTTCTTCCAGCAGTGGACGGGTACAAATGCCATC
AACCTTTATAGTCCCGAAGTATTCCGTCATCTTGGAATCCATGGCAYCAGCK
RKGMWSTCTTCSCTAMTSGTGTTTATKGMGTGGTRMAGGTTGTTTCAGTTG
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TTTTGGTGGTATCGGCCAAGCACTTATGATGTTTTGGTTGGGAGGTTATAGT
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Promoter region (approx. 1000 bases upstream of ORF):
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CTTTTCAATTCGGGCGTTGCGTCTCCCAGGTGCCTGTTAAAAGGGCGAACTT
TAGTGTAATTGTACTAACACAGTCCCTCGGGCTGAGCTCTATTCATC

Terminator region (approx. 700 bases downstream of ORF):
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CGCTCTCGAACCGGGTCAGATGCAATTTTGGTCAGCAATGGTTTATGTTGTT
TCCCCGTAAGTATGCACTAGAAGAGAACAAAACGTCACTATTTGCTCAATG
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CTCSTAATGTCTTCCAGAAAATGGCTTCCC

2. *rafe*:

cDNA sequence:
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Open reading frame coding for predicted protein highlighted.

Genomic Sequence:

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Promoter region (approx. 1000 bases upstream of ORF):
CTGCGAATTGGCATAAGCACTTRAACTTTCGTCTTCCTCACTCTCTTCAGGA
GATTGAGACTGCATCGGTGCAAGCGAGGGTTGCCGTACCGCCCTTTGAGAC
CCCGAAACAAGGGATTCGTCAAGAGCATCAAGAAGACTAGAACGACCGGC
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CCTTAGTCTTTCCTTCTCCCCCTTTCCTTCTTCACTTCACCTTGCTCTAACCGA
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Terminator region (approx. 1200 bases downstream of ORF):
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GTCATGACGTTATATAGATCACTTACATAGATCTTCAGGTTTTCGTAGATCG
ACGACCGACGCTCTTAAATATTTATTTACAGTTTTTCTGTTTTTTGTTTTATT
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3. *mag2*:

Partial cDNA sequence:

CCTTGCCGTTTTCCAGAAGCTGCGACCAGTCCTCGGAGGAGGGGGAACTAA
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ATGATGAGAACGAGGGGTACGATGAGGACGGCCCATCTGAATTGACGGCCA
ACGCGACGTTTCCGGTCGTGAGAGGACATGGCAAAGGAGACGGGGGGAGG
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CCGTCGGAGTGCTACCATAATGGCATAATAAAATTATACTCAGCTGAAAAA
AAAAAAAAAAAAA

CLAIMS:

1. A filamentous fungus transformed with a heterologous sequence of DNA, the fungus being capable of expressing the heterologous DNA, characterised in that the heterologous DNA is under the control of a filamentous fungus transcription promoter active substantially only during stage 1, or later, of the development of the fruiting body of the fungus.

2. A fungus according to claim 1, which is *A. bisporus*.

3. A fungus according to claim 1 or 2, wherein the promoter is that of *abst1*.

4. A fungus according to claim 1 or 2, wherein the promoter is that of *rafe*.

5. A fungus according to claim 1 or 2, wherein the promoter is that of *mag2*.

6. A fungus according to any preceding claim, wherein a selectable marker is linked with the heterologous DNA.

7. A fungus according to any preceding claim, wherein the heterologous DNA is native DNA.

8. A fungus according to any preceding claim, wherein the heterologous DNA is selected such as to affect characteristics of mushroom crop production.

9. A fungus according to any preceding claim, wherein the heterologous DNA encodes: antibodies, including other diagnostic material; secondary metabolites, such as lectins, pesticidal compounds such as *Bacillus thuringiensis* toxin (Bt toxin); therapeutic compounds such as vaccines, steroids, heterocyclic organic compounds; biological macromolecules, such as interferon, endostatin and insulin; and medical enzymes, such as thrombolytics and cerebrosidases.

ABSTRACT

SELECTIVE EXPRESSION IN FILAMENTOUS FUNGI

Gene promoters activated only during development of the fruiting body of filamentous fungi permit expression of large quantities of heterologous genes, even where such are otherwise toxic to the organism.

Gene expression during mushroom development (VM – vegetative mycelium and seven key stages)

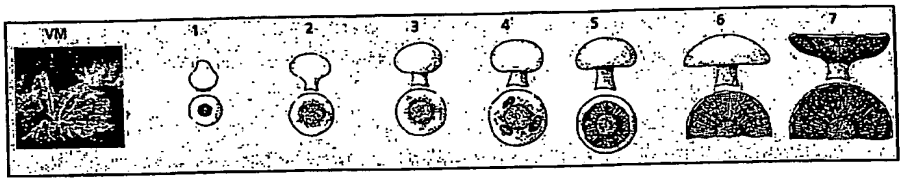
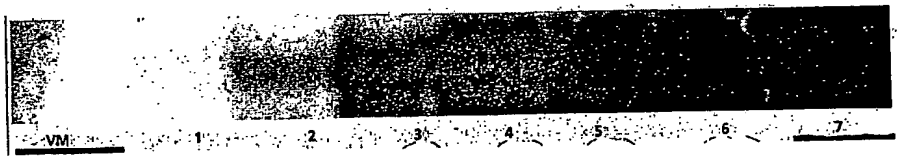
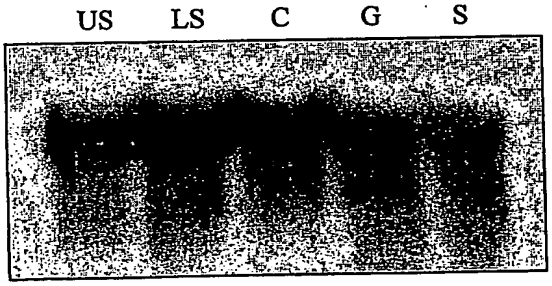


Fig. 1

Gene expression during mushroom development (VM – vegetative mycelium and seven key stages) of *abst1*



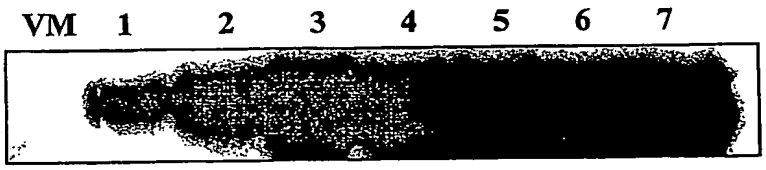
Gene expression in mushroom tissue



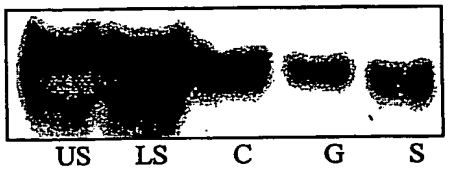
US, upper stipe; LS, lower stipe; C, cap; G, gill; S, skin

Fig. 2

Gene expression during mushroom development (VM – vegetative mycelium and seven key stages) of *rafe*



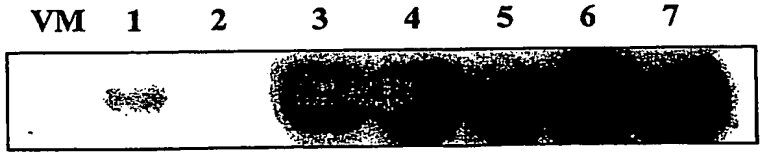
Gene expression in mushroom tissue



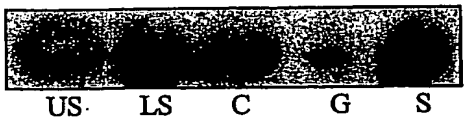
US, upper stipe; LS, lower stipe; C, cap; G, gill; S, skin

Fig. 3

Gene expression during mushroom development (VM – vegetative mycelium and seven key stages) of *mag2*



Gene expression in mushroom tissue



US, upper stipe; LS, lower stipe; C, cap; G, gill; S, skin

Fig. 4